

/THE USE OF TISSUE CULTURE IN
SOYBEAN BREEDING/

by

JAMES TROY WEEKS

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Approved By:

W.T. Schlegel
Major Professor

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LITERATURE REVIEW

Somaclonal Variation and Genetic Variability

In recent years, major advances in research have allowed plant breeders to use tissue culture as a potential means for creating new variability and diverse germplasm for plant improvement (Shepard, 1980; Larkin et. al., 1985). In a plant breeding program, tissue culture could be employed to provide useful variability for mutant selection for economic traits (Hungtu Ma, 1987). Somaclonal variation is genetic variability which is generated during tissue culture (Roy, 1985). To increase and create genetic variability (somaclonal variation) in tissue culture, mutations can be induced by the media plus its components, genotype and explant material. Natural variation can be explained by spontaneous mutation expressed through recombination and natural selection. By inducing mutations, we not only see the natural mutations, but also increase the probability of recovering mutants. When higher plant cells or tissues are induced to proliferate in vitro for even a brief period of time, mutations occur more frequently than expected. (Orton, 1984; McClintock, 1978).

By applying the appropriate selection technique, tissue culture offers many advantages and benefits over natural population mutations and selections. These

advantages include low cost, small area and controlled environments. Brock (1980) discussed the probabilities of obtaining an individual with the desired mutation from a population. The average mutation rates ranged from 1×10^{-2} for chromosomal changes, 1×10^{-3} for a single recessive, and 1×10^{-6} for a single dominate mutation. Brock calculated that at least 4,600,000 genotypes would need to be screened to recover a single dominate mutation for a given trait. The number of genotypes needed to be screened is clearly unworkable at the whole plant level, but is easily attainable in cell culture. Somaclonal variation is a phenomenon that could be potentially useful source of genetic variability for plant improvement (Larkin and Scowcroft, 1981). They define a tissue culture cycle as a process that involves the establishment of a "dedifferentiated cell or tissue culture under defined conditions, proliferation for a number of cell generations, and the subsequent regeneration of plants." Somaclonal variation may find its greatest application for plant improvement with desirable mutations at the cellular level (Scowcroft and Larkin, 1982). Many agronomically important attributes may be screened at the cellular basis. These include resistance to host-specific toxins, tolerance to salinity, metal toxicity, herbicide tolerance, tolerance to temperature stresses and waterlogging.

Somaclonal variation has been noted among important agronomically crops species such as sorghum (Hungtu Ma et. al., 1987), oats (Cummings et. al., 1976), wheat (Cooper et. al., 1986; Larkin et. al., 1984) and rice (Sun et. al., 1983).

Enhanced Variation in Interspecific Hybrids

The genus Glycine Willd. is composed of two subgenera, Glycine and Soja (Moench) F. H. Herm, (Hymowitz and Newell, 1981). Several wild perennial species belong to the subgenus Glycine native to Australia and possess diploid chromosome numbers ranging from thirty-eight to eighty. The cultivated soybeans belong to the subgenus Soja, annual and diploid, have a chromosome number of forty. The perennial species may contain several useful genes for desirable traits such as tolerance to adverse soils, herbicide tolerance, temperature stresses, waterlogging, disease resistance, and host-specific toxins (Larkin and Scowcroft, 1983). It was reported (Burdon and Marshall, 1981) that several of the perennial soybean species showed resistance to leaf rust caused by Phakospora pachyrhizi.

These desired traits could be agronomically beneficial if they could be incorporated from the wild perennial species into the cultivated annual species. One way to

transfer these genes is to make an interspecific cross between the two species and then backcross the hybrid to the annual parent until it resembles the recurrent parent in most characteristics and still contain the desired trait. Evans et. al., (1984) outline a backcrossing procedure using an interspecific hybrid to create genetic variability and selection for improved gene combinations among variants at a rapid pace for a conventional plant breeding program. This type of breeding program has been demonstrated successfully in many crops, such as wheat for several desired traits (Sharma and Gill, 1983; Gill and Raupp, 1987).

Intersubgeneric (interspecific) hybrids have been successfully obtained in our laboratory as well as others (Newell and Hymowitz, 1982; Singh and Hymowitz, 1985). These hybrids were recovered by using in vitro embryo rescue procedure before pod abortion. Unfortunately, in all cases, the recovered hybrid plants from culture have been completely sterile with no seed set.

Tissue Culture and Chromosome Stability

Variability observed in somaclones may be caused at least in part by chromosome breakage and reunion events. If tissue culture generates an environment for enhancing such events, it may provide the means for obtaining the

genetic exchange needed between two genomes in an interspecific hybrid. In an interspecific hybrid it often proves difficult to obtain any pairing and subsequent exchange between the crop and alien chromosomes. Tissue culture may produce a means by which the hybrid material may be able to enhance the frequency of the requisite exchange (Larkin et. al., 1981).

One of the basic aspects of somaclonal variation is that it is a product of chromosome number and structural variation. Many crop species undergo some degree of chromosome instability as a result of going through a tissue culture regime (Evans et. al., 1984). Structural chromosome variation includes deletions, duplications, inversions, and translocations (Orton, 1983). These types of variations have been reported in hybrids of many crop species including soybean hybrids (Ahmad, et. al., 1977, 1979). Numerical chromosome variation involves changes in the number of whole chromosome sets (polyploidy) and changes involving part of the chromosome set (aneuploidy). One must keep in mind that the frequency in which regenerated plants with chromosome changes is influenced by the genotype and ploidy of the starting material as well as the culture method used. In the early years of tissue culture, there were reports of polyploidy in a crop species (Murashige and Nakano, 1967) which has continued to be the

most frequent type of variation (Skirvin, 1978).

As discussed earlier, the major constraint of the soybean interspecific hybrid is sterility. One possible reason for the infertility of the hybrid may be related to spatial separation of parent genomes which could prevent pairing of homologues during meiosis. Finch et. al., (1981) crossed Hordeum distichum x Secale cereale to produce an interspecific hybrid. They encountered the problem of the normal hybrid plantlets dying before they could be transferred to soil. But when they put the hybrid through a intervening callus phase they recovered somaclonal hybrids that grew and tillered vigorously. An interspecific cross was made between Glycine tomentella and G. max by (Singh and Hymowitz, 1985). They recovered the interspecific hybrid and performed cytological analyses on flower buds undergoing meiosis. Four bivalents and 51 univalents were identified during meiosis in the hybrid cells. Also, a chromatin bridge involving a paracentric inversion and chromosome separations in anaphase I cells of meiosis was observed. It was suggested the reason for the interspecific hybrid being completely sterile and having no seed set was a result from the disturbed meiotic stages that subsequently caused shriveled anthers and the absence of stainable pollen. From this and other reports, it seems feasible that passing the soybean interspecific hybrid

through callus culture may be beneficial in the induction of morphogenesis or increasing fertility.

Karyotypic Analysis of Callus Cultures

It is thought that conducting karyotypic analyses of chromosomes in tissue culture would be beneficial. Reasons for karyotypic analyses of cultured cells would be the monitoring of occurrence, degree, distribution, and classes of gross chromosomal changes and variability. By observing the chromosomes of cultured cells over a period of time it may be possible to detect the occurrence of aneuploidy or polyploidy taking place. Preliminary observations of aneuploidy and polyploidy (Orton, 1979) in tissue cultures of Hordeum suggested their use as potential sources of genetic variability. It has been reported in many crop species and in the interspecific hybrid that the length of the culture phase prior to regeneration appears to influence the degree of variation recovered (Kasperbauer et. al., 1979; McCoy et. al., 1981, 1982; Wenzel et. al., 1979). Evidence indicates that the longer the callus is subjected to a culture phase, more variation will occur (Scowcroft and Ryan, 1986). However, caution must be taken because there seems to be a relationship between the length of callus culture and the ability to regenerate a plant. Murashige and Nakano (1967) have shown that extended

cultures can lead to a reduction in morphogenic potential and is correlated with increasing aneuploidy.

It is important to develop a consistent methodology for karyotypic analysis of soybean cultured cells; this would involve the selection of callus material and the appropriate staining protocol. To date there have been few published reports concerning staining protocols and karyotic analysis of cultured cells. Krikorian et. al., (1983) suggested one of the most difficult problems is to extend techniques used on intact plants or plant organs such as root tips to cultured tissues and cells. As techniques and methods are established, more information can be obtained on gross chromosome change and karyology of soybean cultured cells will be easier. Along with monitoring changes in karyology and chromosome behavior in cultured tissue, chromosome counts can also be taken.

Regeneration

In order to successfully recover the desired variation from callus culture a technique for the regeneration of fertile plants must be developed. If no plants can be regenerated that will express the variation at the whole plant level then all previous work of the callus culture in vitro will have been wasted. The selected characteristic will have to be able to pass from one generation to the next. It is understood in cells of higher plants that they

possess the potential for totipotency i.e. the ability of single cell to regenerate into a complete plant (Mitra, 1985). The problem with some crop species is knowing which factors induce the cells to carry out this process. Many factors must be dealt with when trying develop embryogenesis or organogenesis and regeneration of plants from a tissue culture. Some important factors include the explant, genotype, media plus constituents and culturing of tissue with time affecting most of these factors in some way. An explant is a piece of tissue or organ which is removed from the plant for purposes of culture. Success in culturing the explant is influenced by a number of factors inherent to the explant including the genotype of the explant, the size of the explant, physiological age, and the tissue or organ source of the plant. Recent literature indicates the most widely used sources of soybean explant for callus growth and regeneration has been cotyledons and immature embryos (Ranch et. al., 1985; Wright et. al., 1986; Barwale et. al., 1986; Li et. al., 1985).

It has been reported in soybeans and other crop species of variation associated with genotype and degrees of differentiation of callus growth and regeneration (Beverdorsf and Bingham, 1977; Sears and Deckard, 1982). Due to lack of published reports on research of regenerating soybean plants, no superior genotypes have

been identified. However, success in the technology and application of in vitro methods has been achieved because a better understanding of the nutritional requirements of cultured cells and tissues has been developed (Gamborg and Skyluk, 1981). There are several essential nutrients and components in the media that have a direct influence on the performance of callus growth and the ability to regenerate. The media usually contains inorganic salts, an energy source, vitamins and possibly a phytohormone (growth regulator). The nutrients and components of a media will vary in the amount, type and concentration according to the objective of the experiment and the crop species used.

In order to successfully obtain fertile soybean plants, a callus culture medium must be established. In the last few years, several laboratories have developed a technique for successfully regenerating soybean plants from tissue culture (Ranch et. al., 1985; Ghazi et. al., 1986; Wright et. al., 1986; Li et. al., 1985). Each of these articles used a different method to regenerate soybean plants. Barwale et. al., (1986) reported a 65% and 100% plant regeneration via embryogenic and organogenic pathways, respectively, using 54 genotypes from immature soybean embryos plated on the appropriate medium. There has also been reports in literature reporting regeneration of the wild perennial soybean species (Grant, 1984; Widholm

et. al., 1983; Hymowitz, et. al., 1983). From these reports it may be possible then to modify a medium that would allow the regeneration of an interspecific hybrid soybean plant from tissue culture.

Toxin Selection

Being able to regenerate soybean plants from callus culture enables tissue culture selection schemes to be used in a breeding program. One such scheme would be to use tissue culture for the potential development of novel disease-resistant germplasm. This would involve selecting plant cells in culture for resistance to a pathogen toxin and the regeneration of plants from the cultured cells that would have an altered response to the infection by the pathogen. Hartman et. al., (1984) reported alfalfa selected for resistance to culture filtrates of Fusarium oxysporum f. sp. medicaginis in the field showed high levels of resistance and this resistance was stable and highly heritable when transmitted to progeny.

One soybean disease that is of concern to soybean plant breeders is charcoal rot. Charcoal rot (summer wilt, dry-weather wilt) appears mostly in dry, hot climates. The pathogen attacks the plant throughout the season causing debilitation of the host. It seems to be nonhost-specific and is widely distributed in soils. In tropical countries

where the pathogen causes a blight of emerging seedling, plant death up to 77% has been reported (Sinclair, 1982). The effects of the disease to crop species are lower yields and reduced seed quality. By isolating the toxin from Machrophoma phaseolina (Dhingra and Sinclair, 1974) and selecting callus resistant to the toxin, regeneration of plants from the culture resistant cells may develop a disease-resistant cultivar to charcoal rot. In a tissue culture study performed by (Gray et. al., 1986), pathogenic isolates of Phialophora gregata causing brown stem rot in soybeans were used. They reported that the isolates caused browned callus, decreased growth and reduced TTC assay reduction rate in the callus cultures. This would indicate that a toxin selecting scheme may be useful in developing resistant mutants in culture to charcoal rot disease.

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CHAPTER I.

KARYOTYPIC ANALYSIS OF CALLUS CULTURES
DERIVED FROM IMMATURE SOYBEAN EMBRYOS

ABSTRACT

Karyotypic analyses were performed on callus cultures derived from immature embryos from Glycine max (L.) Merr., Glycine tomentella Hayata and their intersubgeneric hybrid. Observations were made to monitor chromosomal variability that existed during the callus culture phase. Embryo size versus survival on the callus inducing medium was compared. It was found from studies of both parents that the size of the intersubgeneric embryos were too small for survival and callus formation on the callus induction medium. A pre-culture on hormone-free medium was first required before the embryo(s) could be transferred to the callus induction medium in order to obtain callus for karyotypic analyses. Karyotypic analyses of Glycine max (cv. Bay) in culture revealed mitotic activity with metacentric chromosomes in cells which could be counted easily. Mitotic activity of callus cells from Glycine tomentella also had metacentric chromosomes and could be consistently counted. It was more difficult to locate mitotic activity in the intersubgeneric callus cells. There was a large degree of variation in the number of chromosomes from one cell to another. The chromosomes in some of the callus cells were atypical; they appeared to be acentric fragments. Because of these chromatin fragments/segments, it was not known what variation was taking place between the two parent genomes.

Therefore, the segments made chromosome counting impossible.

Additional index words: Glycine max, Glycine tomentella, intersubgeneric hybrid, karyotypic analyses, mitotic activity, callus cultures, chromatin material.

INTRODUCTION

The genus Glycine Willd. consists of two subgenera, Glycine and Soja (Moench) F. S. Herm, (Hymowitz and Newell, 1981). Evidence from literature suggests that these two subgenera are partly phylogenetically related. Interspecific crosses between the two subgenera would allow a potential source of new genes for plant breeding work. Although an intersubgeneric hybrid can be obtained, it requires an in vitro rescue procedure with the recovered embryo placed on an appropriate medium for survival because of the small size and immature age.

Very few cytological studies have been conducted in the genus Glycine because of the difficulty of studying plants with a large number of small chromosomes (Ahmad et. al., 1977). Karyotypic and cytological studies of both subgenera, as well as the intersubgeneric hybrid, would provide valuable information concerning the possibility of gene transfer between the two subgenera. Cytological analyses were performed by Singh and Hymowitz (1985) on flower buds of an intersubgeneric hybrid between Glycine tomentella and G. max. They identified four bivalents and 51 univalents during meiosis in the hybrid cells. Chromosome separations and chromatin bridges could also be detected with the disturbed meiotic stages resulting in shriveled anthers and the absence of stainable pollen.

They suggested that gene transfer between the two subgenera Glycine and Soja is feasible and gene exchange is possible at the tertiary gene pool level.

Somaclonal variation occurs frequently in tissue culture (Evans et. al., 1984; Orton, 1983; Ahmad et. al., 1979). Krikorian et. al., (1983) reported that cultured plant tissues and cells that have gone through the callus state are very likely to deviate from the norm in their chromosomal complement. Somaclonal variation occurring in the tissue culture cycle may be beneficial in restoring the fertility of a regenerated hybrid soybean plant. Orton (1980) suggested that in cultures of sterile interspecific hybrids, such as Hordeum vulgare x Hordeum jubatum, it may be possible to isolate and regenerate fertile or novel genetic variants. Cooper et. al., (1978) demonstrated that survival and fertility could be restored when interspecific plants were passed through an intervening callus cycle.

To date there have been few published reports and little research with the karyology of tissue culture cells. Often, it is very difficult to extend techniques used on intact plants or plant organs such as root tips to cultured tissues and cells (Krikorian et. al., 1983). Since interspecific callus was needed for karyotypic studies, a preliminary study was conducted to determine the optimum size (age) of rescued embryos for survival and callus

formation on the culturing media. In this study the primary objective was to monitor changes in karyology and chromosome behavior in cultured cells of soybean callus tissue over period of time. The cells observed will be from G. max, G. tomentella and an intersubgeneric hybrid from both species. A second objective was to develop a consistent and easy method for karyotypic analysis of soybean cultured cells by selecting the appropriate callus material and staining protocol.

MATERIALS AND METHODS

Preparation of explant and parent material for tissue culture. Twenty plants of Glycine max (L.) Merr. (cv. Bay) from the subgenus Soja (Moench) were grown in 2.5 l pots containing a 50:50 mixture of peat to soil. These soybean plants were referred to as a crossing block and were replanted every two weeks for crossing purposes. Eight plants of Glycine tomentella (P.I. 339.657) from the subgenus Glycine were grown in 6.0 l pots containing a 50:50 mixture of peat to soil, these plants are perennials and did not require additional plantings. The plants were grown in the greenhouse starting in August and continuing through November. Greenhouse temperatures were maintained at approximately 21°C during the night and 25°C during the day. Supplemental greenhouse lighting was provided by Sylvania 1000 watt, high-pressure sodium, Maxi-Grow Batwing lamps and maintained for a 14 hour photoperiod. Young flower buds of G. max were emasculated with anther removal and directly hand pollinated with the pollen of newly open flowers of G. tomentella.

Procedure for embryo size determination. In a preliminary study, G. max and G. tomentella immature embryos ranging in size from 0.5 to 8.0 mm were placed on four different media (Table 1). Embryos of varying sizes

of the two genotypes were placed on 5 plates of the four different culture media giving a total of twenty plates. A total of 320 embryos were excised, eight embryos of each genotype on one plate.

Procedures for obtaining immature embryos and callus formation. Soybean pods of Glycine max (cv. Bay) were harvested 15 to 25 days after fertilization. The pods were surfaced sterilized in 70% ethanol for 5 minutes and then washed in a solution of 20% commercial bleach, 0.05% Nonidet P-40 (Octylphenoxypolyethoxyethanol) for 10 minutes. Immature embryos ranging in size from 4.0 to 8.0 mm were aseptically excised and dissected by a scalpel. Cotyledonary pieces, ranging in size from 3.0 to 10.0 mm, were placed in 100 x 15 mm petri dishes containing 20 ml of cultured medium. The entire procedure was done in a vertical laminar flow hood.

The tissue culture medium 5MS (Ranch, et. al., 1985) was of a semi-solid (Murashige and Skoog, 1962) medium with 5 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% sucrose adjusted to pH 5.7 before autoclaving. Media were autoclaved at 121°C for 20 minutes. Soybean pods of G. tomentella were harvested 20 to 25 days after fertilization. The procedure for pod sterilization, embryo rescue and culture medium used with G. tomentella was identical to that used for G. max. The G. tomentella embryos

were more mature (aged), ranging in size from 2.0 to 3.5 mm, and were excised and plated on the same 5MS culture medium.

Pods developed from crossing *G. max* x *G. tomentella* were harvested 10 to 20 days after pollination prior to pod abortion. Pod sterilization techniques and procedures were the same as the techniques and procedures for the two parents. The size of the embryo was dependent upon the age of the pod at harvest time. The immature embryos were placed on MS basal medium plus modified Williams and White vitamins (Williams, 1978) being hormone free. After 10 days, the interspecific immature embryos were dissected into 2 to 3 mm tissue pieces and placed on the 5MS medium containing 5 mg/l of 2,4-D. Initiation and multiplication of cultures were maintained in a Percival Model LVL incubator at 24 +/- 2°C with a 12 hour photoperiod. Illumination was supplied by General Electric cool-white fluorescent tubes ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$). All cultures were transferred every four weeks to the same respective fresh 5MS medium.

Protocol for karyotypic analysis of cultured cells.
After four weeks, small sections of callus cultured cells were transferred from the cultured medium to small screw cap vials containing distilled water. The cultured cells

in the vial were given a pretreatment that consisted of incubating the vials at 0 to 1°C for 24 hours. The cultured cells were then transferred to a fixative solution containing one part of glacial acetic acid plus three parts of 95% ethanol. The cells could be preserved in the fixative up to two weeks. For the preparation of slides, small pieces of callus cells were removed from the fixative solution and placed in modified carbol fuchsin (MCF) stain (Kao, 1975) for 5 minutes. A small portion of the stained callus was placed on a 3" x 1" x 1.2 mm slide in a drop of MCF stain with a coverslip placed over it. Karyotypic observation was performed using an Olympus BH-2 light microscope. Photographs were taken at different magnifications using Kodak Ektachrome film.

Table 1. Composition of media used in the study of embryo size determination.

Medium	Composition
5MS	MS basal medium + 5 mg/l 2,4-D [†]
MSB	MS basal medium + B5 vitamins [‡] + 5 mg/l 2,4-D
MS4	MS basal medium + WW vitamins [§] + 5 mg/l 2,4-D
MS4+	MS basal medium + WW vitamins + 3.5 mg/l zeatin + 0.018 mg/l NAA + 5 mg/l 2,4-D

Note: All were adjusted to pH 5.7 prior to autoclaving.

[†] MS major and minor salts prepared according to Murashige and Skoog (1962) modified with 2,4-D (Ranch et. al., 1985).

[‡] B5 vitamins prepared according to Gamborg et. al., (1968).

[§] Williams and White's vitamins prepared according to Williams (1978).

RESULTS AND DISCUSSION

Determination of Embryo Size

Several callus initiation media (Table 1) were tested to determine the optimum size of the immature embryo plated to its survival and its ability to produce callus on a specific medium. In this preliminary experiment, varying sizes of immature embryos ranging in size from 0.5 to 8.0 mm from G. max and 0.5 to 3.5 mm from G. tomentella were separately placed on each of the four media. It was observed in this experiment that immature embryos from G. tomentella ranging in size from 0.5 to 3.0 mm turned from a dark green to a white color on the four different medias. They did not form any callus and the embryonic tissue appeared to have died. G. tomentella embryos that measured greater than 3.0 mm turned a light green in color and did show signs of callus initiation. Immature embryos from G. max of size 0.5 to 2.5 mm turned from a dark green to pale-yellow, almost white in color and did not form any callus in any of the four media. The embryonic tissues appeared to be dead. However, the larger sizes of embryos (>3mm) from G. max remained a light green color and showed signs of callus initiation in all of the four media. It appeared that the larger the size of the G. max embryo, the more it retained the dark green color and more initiation or formation of callus was produced. In this study it was

believed that the different media composition was not a factor in the survival and callus initiation of immature embryo tissue, rather the size of the immature embryo at plating time.

Results from this experiment provided useful information of possible problems that could be expected in our other studies. The size of the immature embryo for each species is correlated with the age in which it is excised from the ovule. To increase the chances of obtaining the largest size of interspecific embryos that was possible, *G. max* was used as the female and *G. tomentella* as the male parent in an intersubgeneric cross. It was thought since *G. max* produced much larger pods than *G. tomentella* it would also produce larger embryos. Unfortunately, the time at which the intersubgeneric pods can be harvested can not be chosen because of pod abortion ten to twenty days after pollination. In almost all cases the interspecific embryo that was rescued measured less than 3.0 mm in size. The size of the embryo was too small for callus initiation and tissue survival on the medium containing 2,4-D. To correct this problem, interspecific embryos were rescued and placed on a hormone free medium (4MS), without 2,4-D for ten days. The 4MS medium had been successful for the recovery of rescued interspecific soybean embryos (Coble, 1985). They were then dissected

into small pieces and placed on the 5MS media for callus initiation. This procedure was successful in obtaining callus formation and tissue survival from interspecific immature embryos (Fig. 1.A.).

Karyotypic Study

After four weeks on the 5MS medium, callus cultures of G. max (cv. Bay) were observed with a light microscope. The callus growth was slow with little indication of rapidly dividing cells. The composition of the callus was white loose callus along the edges and border, with a more dark friable callus towards the center. It was suspected that the loose-watery callus along the edges would be most recent and therefore cells within this section of callus would be dividing more rapidly. It was this callus that was selected for slide preparations. The loose callus was stained and when squashed onto the slide revealed a desirable single layer of callus cells. The callus cells varied in size and shape, but were frequently large in size with a long rod shape (Fig. 1.A.). In most of the cells, the nucleus was evident by the dark staining color. But, in all slides of this type of callus there were no cells observed in a mitotic cycle. The cells appeared to be in interphase and non-dividing.

Callus was then selected from the area more towards

the middle. This callus was darker in color and more of a dry friable type. It was difficult to get a single layer of cells that were spread out. The cells seemed to be attached to each other and were hard to break apart without damaging the cell wall. When these callus cells were examined under a microscope they appeared shorter in length with a more square cell shape. Almost all cells contained a dark stained nucleus. Mitotic activity was observed in this type of callus (Fig. 1.B.). There were several cells showing various phases of the mitotic cycle. The chromosomes were metacentric and forty chromosomes could be counted in some cells. Polyploidy, aneuploidy and other chromosome abnormalities could not be confirmed in the Bay callus cells that were observed. Center sections taken from the callus mass did not reveal any mitotic chromosome activity and were very difficult to squash.

After four weeks, callus cultures of G. tomentella were studied using the same technique and procedures performed on Bay callus cultures. The relationship between the size, shape, and mitotic activity of cells and the area where the callus was obtained for slide preparation remained the same for the two species except for the number and size of the chromosomes. G. tomentella had eighty chromosomes and they were smaller in size compared to those of G. max. They also were metacentric in chromosome shape

with no chromosome abnormalities visible in the observed cells.

After mitotic activity could successfully be found in each of the parents, callus cultures of the interspecific tissue were examined. Callus sections were selected from all parts of the callus mass. The size and shape of the cells in the interspecific callus tissue had a wide variation throughout the callus mass and were not consistent with those found in cells of both parents. Mitotic activity was found in a few cells, but not defined to any particular area of the callus mass which made it difficult to pin-point an area that was consistent with a high frequency of dividing cells. The most interesting phenomenon of the interspecific callus cells was the wide degree of chromosome variation within a single slide preparation. In a single slide preparation of interspecific callus cells, observed cells had as few as three and greater than sixty masses of chromatin material (Fig. 1.C.). In other cells, mitotic activity was taking place but no mitotic phase could be defined. The cells contain chromatin material or fragments (Fig. 1.D.). These fragments could not be considered true chromosomes because they ranged in size and did not appear to be metacentric chromosomes. However, they had to be chromatin material because of the staining property. Chromosome counts could

not be taken on these cells. It was thought that a possible explanation for these fragments is unequal replicating time for the two genomes during mitosis.

In conclusion, karyotypic analysis used to monitor chromosomal variation in interspecific callus cultures may prove to be very difficult for several reasons. The most significant problem being the high frequency of variation in both chromosome number and chromosome material within a single slide preparation. These results indicate a high degree of variation taking place in culture and one cell would not be a true representation of the whole callus mass. Because there are very few cells dividing and each dividing cell has a different chromosome complement, one lone abnormality can not be defined for the culture cycle. Another problem is the location selection of callus material for karyotypic study. Soybean callus grows at a slow rate which gives a low percentage of dividing cells at the right stage. There is no indication in the callus mass where to find those few dividing cells. But, through future advances and understanding of chromosomal variation of callus cultures many of these problems can be solved allowing for further studies to be conducted.

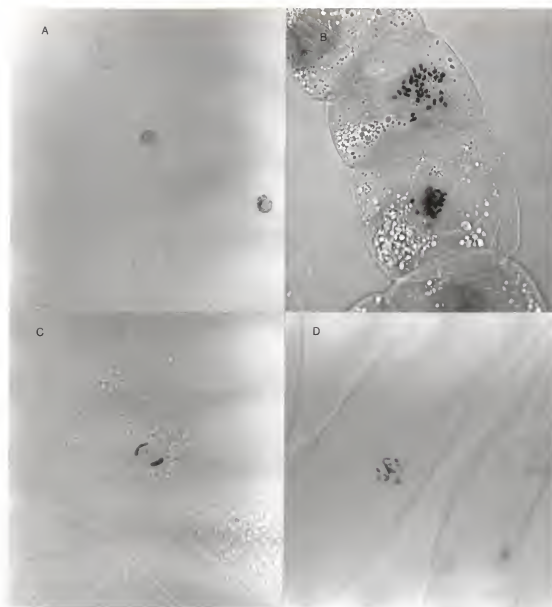


Fig. 1.A.-D. Karyotic variability in tissue cultures of Glycine.

- A. Elongated Glycine max (cv. Bay) callus cell (400X magnification).
- B. Glycine max (cv. Bay) callus cell containing fourty chromosomes (1000X magnification).
- C. Interspecific callus cell containing three masses of chromatin material (1000X magnification).
- D. Interspecific callus cell containing chromatin material, segments and pieces (1000X magnification).

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CHAPTER II.

SOMATIC EMBRYOGENESIS OF GLYCINE MAX FROM EMBRYO-DERIVED TISSUE CULTURES

ABSTRACT

The objective of this study was to develop a routine procedure for the regeneration of viable Glycine max (L.) Merr. soybean plants from tissue culture. Immature embryos excised from soybean pods of six cultivars were placed on three different media to induce callus formation and embryogenesis. Although mature soybean plants were not obtained, somatic-like embryos were produced from callus cultures. The calli derived from the cultivar, Sprite, plated on the LSW medium, yielded the highest percentage of these embryos. These somatic embryos were removed from the callus mass and transferred to various regeneration media in which they continued to grow in size. After several weeks, root formation appeared on some of the somatic embryos. These embryos ceased further development with no shoot production or plants recovered.

Additional index words: totipotency, embryogenesis, culturing environment, somatic embryo, tissue culture, plant regeneration.

INTRODUCTION

Tissue culture has been considered by many to be a potential means for plant improvement and variation. (Shepard, 1980; Larkin et. al., 1985). Tissue culture could provide useful variability and germplasm for mutant selection for economic traits (Hungtu Ma, 1987). To utilize the variability, an approach must first be established to be able to regenerate plants through cell and tissue cultures. Sears and Deckard (1982) reported that predictable plant regeneration from tissue cultures of any crop species is a prerequisite in a plant improvement program. If no plants can be regenerated from the cultured cells to express the variation at the whole plant level, then all previous work in vitro will have been wasted.

Cells in culture possess the potential for totipotency i.e. the ability of a single cell to regenerate into a complete plant (Mitra, 1985). The problem is inducing these cells to embark on a sequence of developmental events to regenerate into a whole plant. Factors that influence the regeneration of a crop species include source of explant, genotype, media plus constituents and the culturing environment of the tissue. By manipulating these factors, several major crops, maize (Lu et. al., 1982), sorghum (Wernicke et. al., 1980), rice (Wernicke et. al., 1981) and wheat (Sears and Deckard, 1982), have been

regenerated from tissue culture.

Until recently, regeneration of complete soybean plants from somatic cells in tissue culture has proven difficult and with limited success while regeneration of other legumes, alfalfa (Bingham et. al., 1975) and clover (Parrot and Collins, 1982) have been accomplished. Recent literature now indicates that several laboratories have developed a technique for successfully regenerating soybean plants from tissue culture (Ranch et. al., 1985; Ghazi et. al., 1986; Wright et. al., 1986; Li et. al, 1985). Barwale et. al. (1986) reported a 65% success in plant regeneration by embryogenesis from 54 genotypes using immature soybean embryos when they were plated on the appropriate medium. It was suggested by (Li et. al., 1985) that development of a technique for regeneration of soybean plantlets from a single cell via somatic embryos is desirable not only for providing new ways to produce soybean varieties, but also for the improvement of existing soybean varieties via modern genetic engineering techniques.

The main objective of this study was to test various media that would first, allow rapid and large amount of callus growth from soybean embryo explants from several genotypes; secondly, regenerate viable, fertile soybean plants consistently and at a high frequency from the embryo-derived callus.

MATERIALS AND METHODS

Sixty plants of Glycine max (L.) Merr. from the subgenus Soja (Moench) were grown in 2.5 l cm pots containing a 50:50 mixture of peat to soil. The plants were a combination of six different cultivars (Bay, Elgin, A3127, Ripley, Sprite, Douglas). Ten pots of each cultivar were grown. The soybean plants were grown in the greenhouse during the winter months from December to February. The temperature ranged 21°C to 26°C during day and night time periods. To compensate for short day length supplemental lighting was supplied by Sylvania 1000 watt, high-pressure sodium, Maxi-Grow Batwing lamps for 14 hours per day. Soybean pods from each of the six cultivars were harvested 15 to 20 days after fertilization. The pods were surfaced sterilized in 70% ethanol for five minutes and then washed in a solution of 20% commercial bleach and 0.05% Nonidet D-40 (Octylphenoxypolyethoxyethanol) for ten minutes. Immature embryos ranging in size from 4.0 to 7.0 mm were aseptically excised with the aid of a scalpel. The meristematic sections of the embryos were discarded and immature cotyledonary segments were retained. The embryos were rescued at different times depending upon the maturity group of the six genotypes. All embryos were rescued within two weeks of each other. Four cotyledonary segments were placed flat side up in 100 x 15 mm petri dishes for

each cultivar containing 20 ml of cultured medium. There were 12 plates of cotyledonary segments for each genotype on the regeneration media. The total number of plates for the six genotypes, on the three regeneration media and four replications, was 72 (Table 2). The entire procedure was performed under sterile conditions in a vertical laminar flow hood. Three different types of culture media, composition outlined in (Table 3), were used in this study. Cotyledonary segments of each cultivar were placed in four plates of each medium. All media were autoclaved at 121°C for 20 minutes. The culture plates were placed in a Percival Model LVL incubator at 24 +/- 2°C with a 12 hour photoperiod. Illumination was supplied by General Electric cool-white fluorescent tubes ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$). Cultures were periodically examined over a four week period. Embryo-like structures were transferred to 5MSD media for regeneration.

Table 2. Results of somatic embryos obtained from embryo plating.

Genotype	Medium (4 plates per)	No. Embryos Plated	Callus Formation (Y/N)	No. Somatic Embryos
Bay	5MS	8	Y	0
"	LS	8	Y	0
"	LSW	8	Y	0
Elgin	5MS	8	Y	0
"	LS	8	Y	1
"	LSW	8	Y	0
A3127	5MS	8	Y	0
"	LS	8	Y	0
"	LSW	8	Y	0
Ripley	5MS	8	Y	0
"	LS	8	Y	0
"	LSW	8	Y	0
Sprite	5MS	8	Y	0
"	LS	8	Y	0
"	LSW	8	Y	5
Douglas	5MS	8	Y	0
"	LS	8	Y	0
"	LSW	8	Y	0
Total	72	144		6

Table 3. Composition of media used for regeneration of soybean plantlets.

Medium	Composition
5MSC	MS basal medium [†] + 20 mg/l 2,4-D [‡]
LS	LS basal medium + 10 mg/l 2,4-D + 0.264 mg/l ABA + 20 g sucrose [§]
LSW	LS basal medium + 5 mg/l 2,4-D + 0.264 mg/l ABA + 0.104 mg/l GA + 20 g sucrose [¶]
5MSD	MS basal medium + 0.5% activated charcoal + 100 g sucrose [#]
SH	SH basal medium + 10 g sucrose ^{††}

Note: All media were adjusted to pH 5.7 prior to autoclaving.

[†] MS major and minor salts prepared according to Murashige and Skoog (1962).

[‡] MS basal medium modified with 2,4-D (J. P. Ranch, personal communication).

[§] LS major and minor salts prepared according to Linsmaeier and Skoog (1965) modified by Ghazi et. al. (1986).

[¶] Modified LS medium (Ghazi et. al., 1986).

[#] MS basal medium modified by J. P. Ranch (personal communication).

^{††} SH major and minor salts prepared according to Schenk and Hildebrandt (1972) modified by J. P. Ranch (personal communication).

RESULTS AND DISCUSSION

Cotyledonary segments from the six cultivars were examined after three weeks on the 5MSC medium. They were swollen and had a light yellow color. There was a small amount of callus growth around the edges of the cotyledons with most of it appearing in the section where the meristematic region had been removed. The callus was of a friable type and creamish in color. No embryo-like structures were found in any of the tissue of the plates containing the six cultivars. The callus continued to proliferate at a slow rate with time but no somatic embryos were observed. The small amount and type of callus may be due partly to the high concentration of 2,4-D in the 5MSC medium.

The cotyledonary segments from the six cultivars that were placed on the LSW responded differently than those on the 5MSC. There was a significantly larger amount of calli proliferation around the edges of the tissue. The callus was more shiny and smooth in structure (Fig. 2.A.) with rapid growth. The callus had a translucent light yellow color which was seen in each of the cultivars tested on the LSW medium. After approximately 3 1/2 weeks of being plated, small pro-embryo like structures were observed in the callus of cotyledon tissue from the cultivar Sprite. These embryo-like structures appeared to be composed of

organized tissue having a green color and were not attached or connected to the callus that surrounded them.

The embryo-like structures were excised from the rest of the callus and placed on 5MSD medium. The 5MSD medium included activated charcoal which was thought to be helpful in removing any 2,4-D from the tissue which might retard further developmental growth of the somatic embryos. On the 5MSD medium the somatic embryos grew in size and acquired a dark green color (Fig. 2.B.-C.) with one somatic embryo appearing to develop both root and shoot structures (Fig. 2.D.). The somatic embryos were subcultured onto the same respective medium each week. After three weeks, the somatic embryos were transferred to hormone-free SH medium. A total of five somatic embryos were obtained from callus tissue on the LSW medium. All somatic embryos were derived from cotyledon segments from the cultivar Sprite. No somatic embryos were found in any of the other callus cultures of the five cultivars on the LSW medium. Of the five somatic embryos that were placed on the SH medium, three of them germinated roots. However, these somatic embryos did not develop further to give any plantlets.

Cotyledonary segments from the six cultivars that were placed on LS medium responded in the same manner as those placed on the LSW medium. The same type of callus was produced but in smaller amounts. One embryo-like structure

was observed in the callus cultures on the LS medium. It was found in callus tissue from the cotyledon segments of the cultivar Elgin. The somatic embryo was transferred onto the 5MSD medium and then to the SH medium. It also did not germinate to give a plantlet after being placed on the SH medium.

As a side study, embryo-derived callus from two interspecific hybrids were placed on the 5MSC and the LS media to initiate embryogenesis. The interspecific callus for this study were obtained from the procedure of interspecific callus production in Chapter I. The callus were left on the two types of media for approximately four weeks. The interspecific callus did not proliferate and no somatic embryos were recovered from either media.

Even though several published reports in the recent years have reported success in regenerating soybean plants, each of them have been different in their protocol and technique for regeneration. This makes it difficult to compare methods and find the best method. Further, reproduction of the methods from the published literature is difficult because important experimental techniques are not always reported. Through personal communication, (J. P. Ranch) mentioned several important factors that may influence the success of regeneration that was not mentioned in the original published reports. These factors

include media modification, size of embryo, orientation of explant on the media and conditions in which plants were grown to obtain the pods for the experiment. These factors may explain the low percentage of somatic embryos obtained in this study. The soybean pods used in this experiment were obtained from soybean plants grown in the greenhouse over the winter months. Although these plants received supplementary light and optimum growing temperature, they did not perform as well as plants grown during the summer months or in the field and may have had an influence on the ability for regeneration.

It is not known why the somatic embryos did not germinate into complete plants. Barwale et. al, (1986) performed histological studies on different stages of somatic embryos in culture. They reported that the almost mature somatic embryo did not possess a clearly defined apical meristem as would be present in a zygotic embryo, but when the somatic embryos were transferred to hormone-free medium they did germinate to produce complete plants. This indicates that the wrong medium may have been used in this study since roots were regenerated in this study. However, somatic embryos were obtained which is the first step in the embryogenesis process. The ease and consistency of regenerating soybean plants will increase as the technique and protocols are refined.

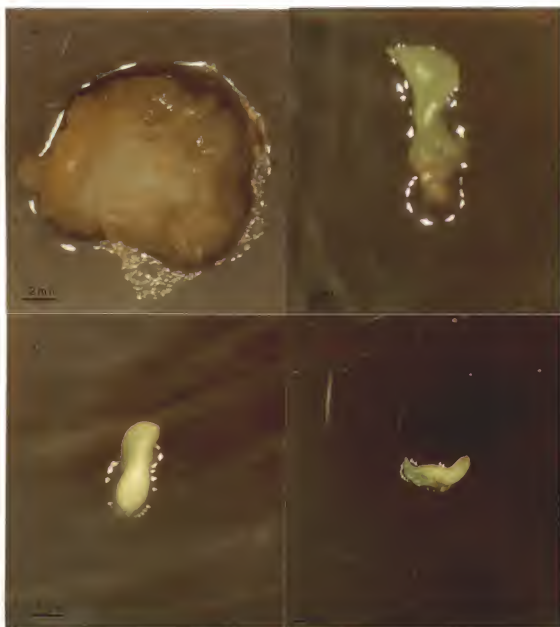


Fig. 2.A.-D. Callus formation and embryoids from embryogenic cultures of soybeans.

- A. Embryogenic callus from cotyledonary segments from cv. Sprite on LSW medium.
- B.-D. Immature somatic embryos on 5MSD medium from cv. Sprite.

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CHAPTER III.

SOYBEAN CALLUS RESPONSE TO TOXIN ISOLATED FROM MACROPHOMINA PHASEOLINA.

ABSTRACT

A procedure was established in which toxin isolated from the fungus Macrophomina phaseolina (Tassi) Goid. was incorporated into a culture medium. Callus from embryo-derived Glycine max (L.) Merr. (cv. Douglas, A2943) were placed on a selection medium. The callus ranged in size and were placed on two levels of toxin concentration, 0.2/20 ml and 0.5/20 ml. Two isolates of M. phaseolina were used in this study, MP01 and MP02. The calli were monitored for their response to the levels of toxin in the selection media. After four weeks, some browning appeared on all sizes of callus of both genotypes in the plates containing 0.2/20 ml and 0.5/20 ml of the toxin. More callus browning was apparent in the 0.5/20 ml toxin plates than the 0.2/20 ml toxin plates. The callus from the cultivar A2943 seemed to show a faster and a larger amount of callus browning compared to the callus from the cultivar Douglas in all toxin plates. The isolate MP02 was determined to have a better specific activity than the isolate MP01.

Acknowledged index words: macrophomina phaseolina, pathogen toxin, charcoal rot, pathogenic isolates, selection medium, resistant mutants, callus response.

INTRODUCTION

As the techniques for regeneration of Glycine max (L.) Merr. becomes more successful, wider use of tissue culture methods will be used in a plant improvement programs. One such method would be in vitro selection of cultured cells for resistance to a pathogen toxin and the regeneration of disease-resistant cultivars. Daub (1986) stated that studies of toxins have been used to select resistant cells in culture and toxin resistance expressed in regenerated plants correlated with significant increases in the levels of disease resistance in the plants. Further, these changes were genetic, as shown by the transmission of resistance to the progeny of the selected plants.

Charcoal rot is a disease caused by the fungus Macrophomina phaseolina (Tassi) Goid. and causes the most damage to soybean crops in hot, dry environmental conditions. The pathogen attacks the plant throughout the season causing debilitation of the host. The fungus is widely distributed in soils and has a wide range of hosts. The effects of the disease on crop species are lowered yields and reduced seed quality. Sim (1980) suggested that charcoal rot is a major disease problem of soybeans in Kansas with the most damage to crops in the eastern one-third of the state.

It has been suggested the only practical way of

controlling the disease is to develop resistant cultivars (Orellana, 1970; Short et. al., 1978). Using tissue culture to develop resistant cultivars would have several advantages over field studies. Because a small amount of space is needed, research would not depend on variable weather conditions and would not be restricted to one plant generation per year. By isolating the toxin from M. phaseolina (Dhingra and Sinclair, 1974), selecting callus cells resistant to the toxin and regenerating plants from the resistant cultured cells, it is thought that a disease-resistant cultivar to charcoal rot may be developed. Gray et. al., (1986) demonstrated that pathogenic isolates of Phialophora gregata caused callus browning and decreased growth of soybean callus in culture. From this report and others it seems feasible that a toxin selection scheme could be useful in developing resistant mutants in culture to charcoal rot disease.

The objectives of this study was to first: develop a technique in which toxin isolated from M. phaseolina could be incorporated into a selection medium at different concentration levels; secondly, monitor the callus tissue response to the toxin. Lastly, the specific activity of the isolates used in the study will also be determined.

METHODS AND MATERIALS

Fifteen plants of Glycine max (L.) Merr. (cv. Douglas) were grown in 2.5 l cm pots containing Redi-mix soil. The soybean plants were grown in the greenhouse during the months of February and March. Greenhouse temperatures were maintained at approximately 21 to 25°C. Supplemental greenhouse lighting was provided by Sylvania 1000 watt, high-pressure sodium, Maxi-Grow Batwing lamps for a fourteen hour photoperiod. Soybean pods from the cultivar Douglas were harvested 15 to 25 days after fertilization. The pods were surfaced sterilized in 70% ethanol for five minutes and then washed in a solution of 20% commercial bleach, 0.05% Nonidet D-40 (Octylphenoxypolyethoxyethanol) for ten minutes. Immature embryos ranging in size from 5.0 to 8.0 mm were aseptically excised by using a scalpel. Cotyledonary segments were placed in 100 x 15 mm petri dishes containing 20 ml of cultured medium. The entire procedure was performed under sterile condition in a vertical laminar flow hood. The tissue culture medium 5MS (Ranch et. al., 1985) consisted of MS basal medium (Murashige and Skoog, 1962) plus 5 mg/l of 2, 4-dichlorophenoxyacetic acid (2,4-D) to induce callus formation. After four weeks, approximately nine pieces of callus sizes ranging from 4 to 20 mm were transferred to plates containing a selection medium. One piece of callus

from the cultivar A2943, approximately 10 mm, was also included in these plates. The selection medium consisted of 20 ml of 5MS medium plus two levels of a toxin (0.5/20 ml and 0.2/20 ml) isolated from Macrophomina phaseolina. Twelve plates were tested for calli response to the two different levels of toxin concentration from two fungal isolates.

The procedure for isolating the toxin was obtained from Dhingra and Sinclair (1974). One isolate of M. phaseolina used in this study, MP01, came from an infected soybean plant in Labette County, Kansas in August 1986. The second isolate, MP02, came from an infected soybean plant in Crawford County, Kansas in September 1986. The toxin was sterilized by using a 0.2 μ M syringe filter and incorporated into 20 ml of the selection medium with a 10 cc sterile syringe after the 5MS media was autoclaved. The specific activity of each isolate was determined by dilution procedure (Pearson, 1982) in which diluted amounts of each toxin were dropped onto a plate containing Luria broth (Miller, 1972) and a lawn of bacteria, Pseudomonas syringae pv. phaseolicola 82-H1. The callus cultures were maintained in a Percival Model LVL incubator at 28 \pm 2°C with a continuous photoperiod. Illumination was supplied by General Electric cool-white fluorescent tubes (60 μ mol m⁻² s⁻¹). Cultures were observed each week for one month.

RESULTS AND DISCUSSION

In the first part of the study, a technique was established to incorporate the toxin into the selection media uniformly and without contamination. It was found that the toxin could be filter sterilized with a 0.2 μ M filter and incorporated into the media without contamination. Optimum size or amount of callus to place on the selection media to give the best response was unknown. It was decided to use various sizes the first time to determine if there were any different responses between them. Results from eight plates indicated that larger pieces of calli responded more quickly to callus browning than smaller ones.

One problem with the toxin was the limited amount that could be obtained by the isolation procedure. From each isolation, approximately 7 ml of toxin was produced which limited the amount that could be added to each plate of callus. Toxin from the isolate MP01 was added to four plates containing 20 ml of selection medium, 2 plates of 0.2/20 ml and 2 plates of 0.5/20 ml. There were also 2 control plates containing no toxin. All four plates that contained the toxin showed callus browning response after four weeks (Fig. 3.). There was greater evidence of callus browning in the plates containing the 0.5/20 ml toxin concentration than the plates of 0.2/20 ml toxin

concentration; callus from both genotypes, Douglas and A2943, responded to the toxin concentrations. A2943 calli which was placed in the center of the plate responded with quicker callus browning compared to the surrounding callus of Douglas on both toxin concentrations.

Selection media containing the isolate from MP02 gave the same results as the isolate from MP01. There was callus browning in both 0.2/20 ml and 0.5/20 ml toxin concentrations with the most in 0.5/20 ml concentration (Fig. 3.B.). Callus from A2943 showed more callus browning response than callus from Douglas. When a comparison was made between the plates of the isolates MP01 and MP02, it appeared the isolate MP02 had more specific activity and produced a more overall callus browning response in the plates containing the toxin.

A dilution study to test the specific activity of the toxin from each isolate was performed. These results would be useful for future experiments in determining the amount of toxin needed for callus response. The isolate MP02 indicated zones of inhibition on the bacteria lawn up to a dilution of 1/32. Because of mixed results, no dilution inhibition zone could be determined for the isolate MP01.

From this study it was concluded that isolated toxin from M. phaseolina could be incorporated into a selection media which caused increased callus browning with increased

toxin levels. Callus derived from A2943 responded more to the toxin in the selection media than the callus of Douglas and the isolate MP02 showed more specific activity than the isolate MP01.

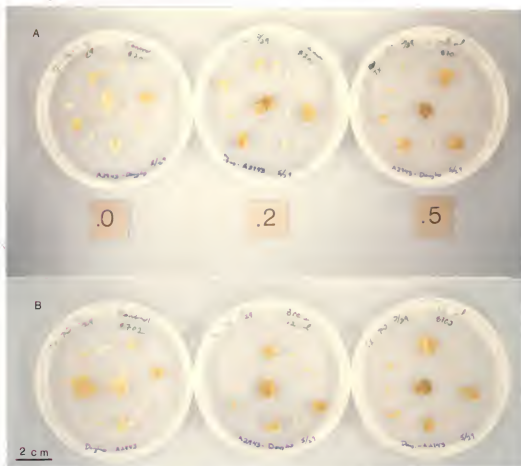


Fig. 3.A.-B. Comparison of different callus sizes from Douglas (outside circle) and A2943 (center) on 20 ml selection media containing toxin levels of 0.2 ml and 0.5 ml isolated from M. phaseolina after four weeks.

- A. Callus response to selection media containing the isolate MP01.
- B. Callus response to selection media containing the isolate MP02.

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THE USE OF TISSUE CULTURE IN
SOYBEAN BREEDING

by

JAMES TROY WEEKS

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Karyotypic analyses were performed on callus cultures derived from immature embryos from Glycine max (L.) Merr., Glycine tomentella Hayata and an intersubgeneric hybrid. Observations were made to monitor chromosomal variability that existed during the callus culture phase between these two species. Slide preparations indicated that chromosomes and mitotic activity appeared normal and frequent within the callus cells of G. max and G. tomentella but was not so evident in the interspecific callus cells. The interspecific callus cells varied in chromosome number and content.

A routine procedure for the regeneration of viable soybean plants from tissue culture was attempted. Immature embryos excised from soybean pods of six cultivars were placed on three different media to induce callus formation and embryogenesis. Although no plantlets were regenerated, six somatic embryos from two cultivars (Sprite and Elgin) on two different media (LSW and LW) were obtained.

Toxin isolated from Macrophomina phaseolina (Tassi) Goid. incorporated at two concentration levels (0.2/20 ml, 0.5/20 ml) into a selection media containing callus pieces from two soybean cultivars (Douglas and A2943). The response of the callus to the toxin was monitored. Results showed callus browning in all plates that contained the toxin. An increase of callus browning was correlated with

an increase of toxin concentration. Also, the callus from A2943 showed more response to the toxin than callus from the cultivar, Douglas.